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Development of Monacolin K-Enriched *Ganghwayakssuk* (*Artemisia princeps* Pamp.) by Fermentation with *Monascus pilosus*

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Monacolin K-enriched *ganghwayakssuk* (*Artemisia princeps* Pamp.) was developed by fermentation with *Monascus* sp. Among the 15 *Monascus* spp. isolated previously from *Monascus* fermentation products, *Monascus pilosus* KMU108 produced 2,219 mg/kg of monacolin K during *ganghwayakssuk* fermentation with no detectable citrinin. The optimum concentrations of *ganghwayakssuk* and glucose determined from the response surface methodology (RSM) design were 2.2% and 3.8%, respectively. By applying these conditions, the monacolin K productivity was increased to 3,007 mg/kg after 15 days of fermentation. On the other hand, other characteristics such as the total content of flavonoids and phenolic compounds, and the antioxidant activity were relatively unchanged. Therefore, *Monascus*-fermented *ganghwayakssuk* is an excellent biomaterial for the development of functional foods because of its high level of monacolin K, known to lower cholesterol levels.

Keywords: *Gangwhayakssuk* (*Artemisia princeps* Pamp.), response surface methodology, *Monascus pilosus*, monacolin K

Mugwort (*Artemisia* spp.) is used in various types of foods or as a food additive because of its unique flavor and taste. Traditionally, it has been used as an herbal medicine to treat gastroenteritis, diarrhea, and uterine hemorrhage [2, 23, 34]. Mugwort has also been used to improve health, nourish the blood, and as a stomach medicine [26]. Currently, it is listed in the Korean Herbal Pharmacopoeia and Korean Natural Drug Standards. *Artemisia* sp. belongs to the family Compositae, of which about 200 species are known to grow in the Northern hemisphere [26]. Approximately 38 species have been reported in Korea and their characteristics are unique to the growing regions [26]. Among them, *ganghwayakssuk* (*Artemisia princeps* Pamp.), growing mainly in Ganghwa Island in Korea, grows up to 70 cm in height and its leaf has the shape of a lion's foot, and is also called *sajabalssuk*. *Ganghwayakssuk* contains several bioactive compounds such as the flavonoids eupatilin and jaceosidin as well as vitamins A, B_1, B_2, and C, and various minerals [14, 27]. In addition, it contains more than 65 essential oils with strong antibacterial activity against *Streptococcus mutans* and *Streptococcus sanguis* [6]. *Ganghwayakssuk* is used widely for traditional *ssuk* products such as tea, rice cake, bread, and natural pigments [29]. *Ganghwayakssuk* has great potentials as functional foods or food additives because of its bioactivities, such as antioxidant, antibacterial, and anticancer effects, which have been verified scientifically [5, 12, 20]. Hwang *et al*. [14] reported that *gangwhayakssuk* extract is effective as an antioxidant in deep fried chicken nuggets.

We have been developing functional food materials by fermentation with the filamentous fungi *Monascus* [13]. *Monascus*-fermented products should have additional bioactive compounds to intrinsic ones because *Monascus* species produce a range of bioactive compounds such as monacolin K, γ-aminobutyric acid (GABA), and dimerumic acid (as reviewed in references [10, 24]). Among the bioactive compounds produced by *Monascus*, monacolin K (also known as mevinolin or lovastatin) has received a great deal of attention because it lowers the cholesterol level in the blood by limiting cholesterol biosynthesis as a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) [8, 9]. In this study, monacolin K-enriched *ganghwayakssuk* was developed by fermentation with newly isolated *Monascus pilosus*. *Monascus*-fermented *ganghwayakssuk* contains additional bioactive compounds

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including monacolin K, resulting in improved functional food materials.

Materials and Methods

Chemicals and Media

Monacolin K, citrinin, Folin–Ciocalteu, and quercetin were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). All HPLC-grade solvents were obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). *Ganghwayakssuk* was supplied by Sanedeule (Ganghwa, Incheon, Korea). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were acquired from Acumedia Inc. (Baltimore, MD, USA).

Strains and Growth Conditions

Fifteen *Monascus* strains, which had previously been isolated from *Monascus* fermentation products [16, 17], and 4 type strains, *Monascus ruber* KCTC6122, *Monascus purpureous* KCTC60169, *Monascus kaoliang* KCCM 60154, and *Monascus pilosus* KCCM60084, which had been obtained from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea) or the Korean Culture Center for Microorganisms (KCCM, Seoul, Korea), were used in the initial screening.

To identify the *Monascus* isolates, internal transcribed sequences (ITS) were determined after PCR amplification using the ITS4 (TCCTCCGCTTATTGATAT) and ITS5 (GGAGTAAAAGTCGTA ACAA) primers [17].

The spores were harvested using a 0.85% saline buffer after culturing each strain on PDA plates for 5 days at 30°C. The spores were inoculated into 100 ml of *ganghwayakssuk* base media (2% *ganghwayakssuk*, 2% glucose) at 2.0×10^4 spores/ml. All broth cultures were incubated for 10 days at 30°C with constant shaking at 150 rpm. The culture broth was freeze-dried in a freeze dryer (Ilshin Biobase, Yangju, Korea).

Optimal Concentration of Nutritional Sources and RSM Experimental Design

For the optimal production of monacolin K in *ganghwayakssuk* fermentation, several nutritional sources known to be effective in other substrates such as rice, soybean, and red ginseng were tested for their effects on monacolin K production. As carbon sources, glucose in the basal medium (2% *ganghwayakssuk*, 2% glucose) was substituted for sucrose, lactose, and galactose. Malt extract, beef extract, and peptone were added as nitrogen sources to the basal medium at 1.5%, 3%, and 4%, respectively [21, 31]. For the inorganic salts, $CaCl_2$ and $(NH_4)_2SO_4$ were added to the basal media at 2% and 1%, respectively [30].

The increase in biomass during fermentation was determined by measuring the dry cell weight. The fermentated samples were filtered using an apparatus made from Kovax-Syringe® (Korea Vaccine Co., Ansan, Gyeonggi-do. Korea) and bandage (KMH Co., Anyang, Gyeonggi-do. Korea) and then washed with distilled water until all materials except for the cells had been removed. The filtered and washed cells were dried to a constant weight at 105°C.

The major variables affecting monacolin K production were *gangwhayakssuk* and glucose. Therefore, these two medium ingredients were chosen for further optimization through RSM. The central composite design (CCD) was carried out to determine the optimum *gangwhayakssuk* and glucose concentrations for monacolin K production.

Measurement of the Antioxidative Activity

One gram of sample was extracted with 100 ml of ethanol for 24 h at 60°C [32]. The extract was filtered through a Whatman No. 42 filter paper (Whatman PLC, Springfield, England) and concentrated in a rotary evaporator (EYELA, N-1000SW, Tokyo, Japan). The antioxidative activity was measured using an *in vitro* assay system against the 1,1-diphenyl-picrylhydrazyl (DPPH) radical [12]. The ethanol extracts were mixed with 800 µl of 0.15 mM DPPH. The DPPH free radical-scavenging activity was determined by reading the absorbance at 517 nm after a 30 min reaction.

Measurement of the Monacolin K, Citrinin, Flavonoid, and Polyphenol Contents

The quantitative analysis of monacolin K and citrinin was carried out by HPLC as described previously [13, 16].

The total flavonoid concentration was determined using a procedure reported elewhere [25]. The ethanol extract (100 µl) was mixed with 0.1 ml of 10% aluminum nitrate, 0.1 ml of 1 M potassium acetate, and 4.3 ml of 80% ethanol. After a 40 min reaction at room temperature, the absorbance was determined at 415 nm. The total flavonoid concentration was calculated using quercetin as the standard.

The content of polyphenols was measured using a slight modification of the method reported by Folin and Denis [11]. One ml of each extract was mixed with 1 ml of a Folin–Denis reagent and 1 ml of 10% Na_2CO_3. After 30 min reaction at room temperature, the absorbance at 760 nm was measured. The polyphenol content was determined by comparison with a standard curve for gallic acid.

Proteins, Lipids, and Ash Analysis

For ash analysis, 1 g of the freeze-dried samples was burnt at 500°C for 15 h in a high-temperature furnace (Samheung, Pocheon, Gyeonggi-do, Korea). The ash content is expressed as the portion of weight remaining after burning [19]. The protein content was determined using a B-324 distillation unit (Bucui Co., Flawil, Switzerland) [18]. The lipids were extracted using Fexika (IKA, Staufen, Germany) and concentrated by drying, and the weight is expressed in % [28].

Results and Discussion

Screening a Strain Suitable for *Ganghwayakssuk* Fermentation

Because *Monascus* sp. produces citrinin, which is both nephrotoxic and hepatotoxic [3], the selection of a strain that produces little or no citrinin is very important when applying this fungus for food products. We had previously isolated 17 strains from *Monascus* fermentation products [16, 17]. The production of citrinin from these 15 and 4 type strains in the *ganghwayakssuk* media was evaluated, after 10 days of culture, by HPLC. Citrinin was produced at various levels ranging from no detection to 2,636.0 µg/kg (Table 1). Isolate H produced the maximum monacolin K at 2,219.0 mg/kg, but with no detectable citrinin (Table 1).

Table 1. Citrinin and monacolin K production in *ganghwayakssuk* by the isolated and type strains.

Strain	Citrinin (μg/kg)	Monacolin K (mg/kg)
Isolate A	ND	32.1 ± 0.0
Isolate B	2,636.0 ± 250.0	1,316.2 ± 52.0
Isolate C	ND	ND
Isolate D	ND	404.3 ± 0.0
Isolate F	52.0 ± 6.0	ND
Isolate H	ND	2,219.0 ± 462.0
Isolate I	ND	89.9 ± 0.0
Isolate J	ND	45.8 ± 0.0
Isolate K	1,756.0 ± 129.0	1,259.4 ± 129.0
Isolate M	28.0 ± 0.0	693.7 ± 142.0
Isolate N	268.0 ± 26.0	1,449.1 ± 232.0
Isolate O	ND	ND
Isolate T	ND	ND
Isolate U	ND	ND
Isolate V	ND	ND
M. pilosus KCCM 60084	ND	ND
M. purpureous KCTC 60169	ND	ND
M. ruber KCTC 6122	84.0 ± 11.0	ND
M. kaoliang KCCM 60154	ND	ND

Each strain was inoculated into 100 ml of base medium (2% *ganghwayakssuk*, 2% glucose) and cultured for 10 days at 30°C with constant shaking at 150 rpm. The values are the mean ± SD. ND, not detected.

The identity of isolate H was determined based on the ITS sequence comparisons, phylogenetic grouping, and morphological observations [17]. The ITS region showed the highest sequence similarity to *M. pilosus* at 98%. In addition, it showed typical *M. pilosus* morphological characteristics. The isolate had globular and hyaline 5–12 μm conidia and produced red globular ascocarp 30 μm in diameter. Therefore, it was designated as *Monascus pilosus* KMU108.

Effect of the Concentration of *Ganghwayakssuk* on Monacolin K and Citrinin Production

To determine the optimum concentration of *ganghwayakssuk*, the base media were prepared with various concentrations of *ganghwayakssuk* ranging from 0.5% to 3% (Table 2). The quantity of monacolin K production increased with increasing *ganghwayakssuk* concentration up to 2%, at which point the amount of monacolin K was 2,079.0 mg/kg. With further increases in *ganghwayakssuk* concentration over 2%, the quantity of monacolin K production decreased and *Monascus* did not grow in more than 5% *ganghwayakssuk*. The inability of growth of *Monascus* on a higher *ganghwayakssuk* concentration may be due to the antimicrobial activity of *ganghwayakssuk*. The essential oils of *ganghwayakssuk* were reported to exhibit antibacterial activity to *Bacillus subtilis* at 10–100 ppm as well as antifungal activity to *Aspergillus nidulans* at ≥1,000 ppm [1]. Citrinin was detected only in culture with more than 2% *ganghwayakssuk*, but its level was low (≤22.1 μg/kg). Therefore, the optimum *ganghwayakssuk* concentration was set to 2%.

Table 2. Effect of the *ganghwayakssuk* concentration on citrinin and monacolin K production.

Ganghwayakssuk (%)	Citrinin (μg/kg)	Monacolin K (mg/kg)
0.5	ND	776.0 ± 38.0
0.75	ND	1,031.0 ± 88.0
1.0	ND	1,638.0 ± 24.0
1.5	ND	1,742.0 ± 457.0
2.0	13.4 ± 2.8	2,079.0 ± 187.0
2.5	22.1 ± 4.3	1,422.0 ± 169.0
3.0	6.8 ± 0.8	1,347.0 ± 490.0

Each strain was inoculated into 100 ml of base medium (2% glucose) supplemented with various concentrations of *ganghwayakssuk*, and cultured for 10 days at 30°C with constant shaking at 150 rpm. The values are reported as the mean ± SD. ND, not detected.

Effects of Carbon, Nitrogen, and Inorganic Salt Sources on Monacolin K and Citrinin Production

Several nutritional sources were added to the *gangwhayakssuk* media to design the proper medium to produce monacolin K-enriched *ganghwayakssuk*. For carbon sources, glucose was most effective, yielding 2,050.0 mg/kg monacolin K (Table 3). Glucose is a good carbon source for *Monascus* sp. growth [33], and the high production of monacolin K might be due to the better growth. As organic nitrogen sources, the addition of malt extract resulted in 830.0 mg/kg of monacolin K production, whereas the addition of beef extract or peptone did not produce any monacolin K or pigments (Table 3). These results agree with a previous

Table 3. Effects of carbon, nitrogen, and inorganic salt sources on monacolin K and citrinin production.

Nutrients	Citrinin (μg/kg)	Monacolin K (mg/kg)
2% Glucose	ND	2,050.0 ± 35.0
2% Sucrose	171.0 ± 45.0	790.0 ± 47.0
2% Lactose	ND	1,010.0 ± 2.0
2% Galactose	ND	730.0 ± 4.0
3% Beef extract	13.4 ± 0.0	ND
4% Malt extract	ND	830.0 ± 29.0
1.5% Peptone	ND	ND
2% $CaCl_2$	ND	1,690.0 ± 5.0
1% $(NH_4)_2SO_4$	ND	ND

The culture media were prepared with different carbon sources. To evaluate the effect of the addition of nitrogen and salt sources, each nutritional source was added to the base medium (2% *ganghwayakssuk*, 2% glucose). The culture was carried out for 10 days at 30°C with constant shaking at 150 rpm. The values are the mean ± SD. ND, not detected.

Table 4. Effect of the glucose concentration on monacolin K and citrinin production.

Glucose (%)	Citrinin (μg/kg)	Monacolin K (mg/kg)
1.0	ND	75.3 ± 47.6
1.5	ND	594.9 ± 166.3
2.0	ND	1,704.9 ± 267.7
2.5	ND	1,987.2 ± 108.4
3.0	ND	2,516.2 ± 30.1
5.0	25.6 ± 2.0	1,255.3 ± 19.4

The values are the mean ± SD. ND, not detected.

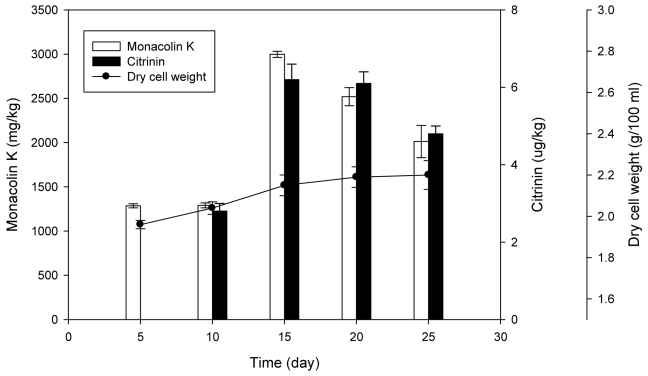

Fig. 1. Monacolin K production during *ganghwayakssuk* fermentation by *M. pilosus* KMU108 in media optimized by RSM.

report showing that nitrogen sources decreased or had no effect on monacolin K production [33]. As inorganic salts, the addition of $CaCl_2$ produced 1,690.0 mg/kg of monacolin K, but $(NH_4)_2SO_4$ did not (Table 3). Most of the nutritional sources did not yield citrinin, except for 2% sucrose, where 171.0 μg/kg citrinin was detected. Therefore, the addition of other than glucose is ineffective in increasing the production of monacolin K-enriched *ganghwayakssuk*.

Among the additional nutritional sources, glucose had positive effects on monacolin K-enriched *ganghwayakssuk* production. To determine the optimum glucose concentration, the quantity of monacolin K production was determined in *ganghwayakssuk* with different glucose concentrations. The level of monacolin K production increased with increasing glucose concentration up to 3%, yielding 2,516.2 mg/kg and decreased significantly when more than 3% glucose had been added (Table 4). Jung and Yu [15] reported that the *Monascus* sp. biomass yield was a maximum at 3% glucose. Therefore, the higher monacolin K production appears to be related to the better growth of *Monascus*. Only 25.6 μg/kg citrinin was detected when 5% glucose was used.

Optimal Medium by the Central Composite Design of the Response Surface Methodology

The central composite design (CCD) of the RSM was used to determine the optimal medium composition for the production of monacolin K-enriched *ganghwayakssuk* [4, 33]. The independent variables were the *ganghwayakssuk* concentration (X_1) and glucose concentration (X_2), which have a positive effect on monacolin K production. The central values (0) were determined to be 2% *ganghwayakssuk* and 3% glucose, whereas the (-1, +1) values were ±1% *ganghwayakssuk* and ±2% glucose, resulting in a total of 9 experimental runs. Table 5 lists the CCD design and corresponding experimental data. Model equation prediction and regression analysis were performed using MINITAB 14. Multiple regression analysis of the experimental data yielded the following second-order polynomial equation.

$$Y = -477.745 + 1782.13*X_1 + 262.306*X_2 - 446.111*X_1^2 - 46.9861*X_2^2 + 47.6250*X_1*X_2$$

(X_1 = *ganghwayakssuk*; X_2 = glucose; Y = monacolin K)

The coefficient of determination R^2 was 0.919, suggesting that a second-order polynomial equation model is sufficient for approximating the response surface of the experimental design. The maximum monacolin K production was predicted to be 1,999.9 mg/kg. The theoretical optimum concentrations were 2.2% *ganghwayakssuk* and 3.8% glucose. Applying the optimum conditions designed by the RSM yielded 2,445.0 mg/kg of monacolin K, which was 623.0 mg higher than the base medium with 4.4 μg/kg of

Table 5. Culture conditions based on the RSM and monacolin K production.

Run No.	Culture condition (%)		Monacolin K production (mg/kg)	
	Ganghwayakssuk	Glucose	Experimental	Predicted by RSM
1	1	1	1,191.7	1,125.3
2	1	3	1,261.3	1,350.3
3	1	5	1,266.7	1,204.0
4	2	1	1,546.0	1,611.1
5	2	3	1,984.0	1,941.3
6	2	5	1,922.0	1,900.3
7	3	1	1,208.0	1,209.2
8	3	3	1,691.0	1,644.7
9	3	5	1,664.0	1,709.0

Table 6. Characteristics of monacolin K-enriched *ganghwayakssuk*.

Constituents	*Ganghwayakssuk*	Fermented *ganghwayakssuk*
Protein (%)	1.88 ± 0.14	0.84 ± 0.02
Lipid (%)	0.62 ± 0.08	0.79 ± 0.11
Ash (%)	2.06 ± 0.12	1.10 ± 0.25
DPPH SA (%)	90.40 ± 2.10	88.90 ± 1.60
Total phenolic compounds (mg/g)	12.92 ± 0.17	12.80 ± 0.20
Total flavonoids (mg/g)	2.77 ± 0.10	2.82 ± 0.08

The values are the mean ± SD. SA, scavenging activity.

citrinin produced after 10 days of incubation at 30°C. Under these conditions, monacolin K production was highest at 3,007.0 mg/kg after 15 days of incubation (Fig. 1).

Characteristics of Monacolin K-Enriched *Ganghwayakssuk*

Monascus-fermented *ganghwayakssuk* had similar characteristics in terms of the general composition as the unfermented one (Table 6). The protein content decreased from 1.88% to 0.84%, which might be due to some protein being consumed during fermentation. In contrast, the lipid contents increased slightly from 0.62% to 0.79%. *Ganghwayakssuk* exhibits free radical scavenging activity ranging from 56.2% to 95% [6]. *Monascus*-fermented *ganghwayakssuk* exhibited 88.9% DPPH scavenging activity, which was similar to the unfermented one. The total phenolic and flavonoid compounds after fermentation were 12.8 mg/g and 2.8 mg/g, respectively. These levels were almost unaffected by fermentation.

In this study, monacolin K-enriched *ganghwayakssuk* was developed by fermentation with *Monascus*. The monacolin K content was as high at 3,007 mg/kg, which was six times higher than the standard (500 mg/kg) for *Monascus*-fermented rice to be claimed as a functional food set by the Korea Food & Drug Administration (KFDA). Normally, the monacolin K levels in *Monascus*-fermented rice or soybean were ≤15,000 mg/kg [22]. Therefore, *ganghwayakssuk* is a better substrate for monacolin K production. The high level of monacolin K production in *ganghwayakssuk* by *Monascus* is due probably to the antibacterial compounds in *ganghwayakssuk,* which may induce monacolin K production. Consistent with this assumption, monacolin K production in red ginseng medium, which exhibits antimicrobial properties, was more than 3,000 mg/kg [13]. The original functionality of *ganghwayakssuk* was unaffected, as indicated by the DPPH radical scavenging activity, total phenolic compounds, and total flavonoids. Considering the health functionality of monacolin K and other bioactive compounds produced by *Monascus*, monacolin K-enriched *ganghwayakssuk* would be useful as a functional food material, particularly for the prevention of cardiovascular diseases.

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